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13. ABSTRACT ***(Maximum 200 Words)***

The goal of these studies was to assess ERRα's utility as a novel breast cancer biomarker. Using real-time quantitative polymerase chain reaction assays, we profiled mRNA levels in random clinical breast cancers (n = 38) and normal mammary epithelial cells (MECs) enriched from reduction mammoplasties (n = 9). These studies showed that: (i) ERRα was overexpressed in approximately 10 % of tumors; (ii) ERRα mRNA levels were significantly higher in progesterone receptor (PgR)-negative tumors (those lacking functional ERα) than in PgR-positive tumors; (iii) ERRα mRNA was more abundant than ERα, ERβ, ERRβ, and ERRγ in PgR-neg tumors; (iv) ERRα expression correlated with ErbB2, ErbB3 and ERβ expression by Spearman analysis; and (v) ERRα expression patterns were ordered together with ErbB2, ErbB3, and EGFR by cluster analysis. Therefore, ERRα may be a target of ErbB signaling. ErbB members signal via the mitogen-activated protein kinase pathway and ErbB2 has been associated with tamoxifen resistance. Hence, the following hypothesis was developed: ERRα, independent of ERα, potentiates transcription of genes whose promoters contain estrogen-response elements in tumors containing high MAPK activity due to overexpression of ErbB members. Thus, ERRα status may indicate sensitivity to hormonal and ErbB2-based therapies.

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TABLE OF CONTENTS

INTRODUCTION

Approximately 50 % of breast cancer patients with estrogen receptor-positive (ER+) tumors respond to antiestrogen therapy (1). Since expression of progesterone receptor (PgR) is dependent upon ERα activity, further selection of patients for ER-positive and PgR-negative tumors enhances the breast cancer hormonal therapy response rate to nearly 80 % (2). The estrogen-related receptor α (ERRα), an orphan nuclear receptor that shares significant sequence identity with ERα and ERβ but does not bind estrogens (3), has been shown to bind and activate transcription through estrogen response elements (EREs) (4) as well as ERR-response elements (ERREs) which are composed of an ERE half-site with a 5' extension of 3 base pairs (4, 5). Hence, ERRα may modulate estrogen responsiveness, making it a plausible candidate for a novel breast cancer prognosticator and target for therapy. First, ERs and ERRα may compete for binding to a response element. Second, ERs and ERRα may selectively bind subelements within a composite element to act in concert. Thus, we proposed to test whether ERRα plays an important role in the development of some breast cancers by modulating or substituting for ER activities. The specific questions addressed by this award are the following: (i) Does expression of ERRα correlate with the expression of known breast cancer prognosticator genes and clinical tumor properties? (ii) Which genes are potentially regulated by ERRα and what are the molecular mechanisms involved in ERRα modulation of estrogen-responsive transcription?

BODY

Specific Aim I - To test whether alterations in expression, RNA splicing, phosphorylation status, subcellular localization, or mutations in ERRα significantly correlate with the development of some breast cancers. (A) Assays will be developed with human mammary carcinoma MCF-7 cell derivatives in Aim II to characterize ERRα RNA and protein abundance, possible splicing variants, possible phosphorylation isoforms, subcellular localization, and possible mutations. (B) These assays will be used to characterize clinical ERα-positive primary breast carcinomas for ERRα. (C) Additionally, ERα-positive/tamoxifen-resistant breast tumors will be examined for ERRα RNA expression, splicing variants, and possible mutations.

Task 1. To test whether alterations in ERRα significantly correlate with the development of clinical ERα-positive primary breast carcinomas and ERα-positive/tamoxifen-resistant breast tumors (months 1-36)

Task 1A. Develop assays with breast cancer cell lines to look for alterations in ERRα (months 1-12).

Task 1A was accomplished as discussed in the year 1 report.

Task 1B. Examine primary ERα-positive breast carcinomas (months 2-24).

I have expanded the scope of Task 1B by broadening the class of tumors examined from only ERα-positive to include ERα-negative tumors as well. Additionally, I had originally proposed to examine approximately 100 tumors, however, the tissue repository (SPORE) at Baylor College of Medicine has been subjected to extensive flood damage due to a hurricane (Gary Clark, SPORE Director, personal communication). Prior to the flooding, I had already received 40 breast cancer samples, 38 of which were suitable for analysis. Unfortunately, I cannot receive additional samples until the tissue repository is re-established, a time period likely beyond the duration of this fellowship. Thus, the experiment proposed in this fellowship will necessarily be limited to those tissues already in hand.

Revised Task 1B Examine random primary breast carcinomas (months 2-24).

To test whether ERRα is involved in breast carcinogenesis, a panel of 38 clinical random breast cancers and normal mammary epithelial cells (MECs) prepared from 9 individuals were characterized for mRNA expression of this gene by real-time quantitative polymerase chain reaction (Q-PCR) assays. Expression of ERRβ and ERRγ was also determined as ERR family members share many biochemical and transcriptional activities. In addition to ERR members, expression of ERα, ERβ, EGFR/ErbB1, ErbB2/HER2/*neu*, ErbB3, and ErbB4 was also determined. These genes were analyzed for alterations in mRNA levels between tissue groups and for correlations of expression between the genes and clinical properties indicative of tumor aggressiveness.

Tissue sources.

The clinical tumor samples were obtained from the National Breast Cancer Tissue Resource (SPORE) at Baylor College of Medicine (Houston, Texas) via a collaborative arrangement with Dr. Gary Clark who also served as the study's biostatisician. Prior to our receipt of the tumors, several clinicopathologic properties of the tumors were measured including ER and PgR protein levels by the ligand binding assay (ER-LB and PgR-LB status, respectively), as well as S-phase fraction and DNA ploidy by flow cytometry. We were blinded to these clinical tumor properties until completion of our studies. The normal MECs were obtained from Dr. Stephen Eithier at the University of Michigan-Ann Arbor and from Dr. Michael N. Gould, a

colleague in our department at the University of Wisconsin-Madison. Because mammary gland is a fatty tissue, it is technically very difficult to isolate high quality RNA from this source unless the MECs are enriched from the bulk tissue. Hence, our laboratory received the normal MECs after Dr. Either's and Dr. Gould's laboratories had isolated these MECs from reduction mammoplasties through a process involving collagenase dispersion of the tissues, differential centrifugation, and filtration steps.

Real-time quantitative polymerase chain reaction (Q-PCR) assays.

To measure expression of ER, ErbB, and ERR family members, real-time Q-PCR assays were developed. In these assays, continuous measurement of fluoresence due to specific complex formation of Sybr Green I with double-stranded PCR products versus single-stranded DNA facilitated accurate and sensitive quantitation of initial mRNA molar amounts (6-8). The assays were developed using an ABI 7700 sequence detection system (Applied Biosystems). Because PCR efficiency decreases with increasing number of cycles due to limiting polymerase, nucleotides and primers, the critical parameter recorded in the real-time Q-PCR assay is the threshold cycle (C_t), *i.e.*, the cycle (measured to a fractional value of the 1 minute extension phase of the PCR) when PCR products are initially detected over background fluorescence. Background fluorescence levels are determined from the signal intensity of no-template control reactions. Spectral compensation of signal intensity differences among sample wells is accomplished by the inclusion of ROX, a fluorescent reporter dye, in the enzyme reaction buffer (Molecular Probes). To minimize non-specific product amplification, the real-time Q-PCR assays employed HotStar Taq (Qiagen), a chemically modified form of Taq polymerase that is completely inactive at room temperature and requires an initial 10-minute heat activation step at 95° C. PCR primers were optimized for high efficiency, *i.e.*, to yield product sizes smaller than 150 bp and no spurious bands. PCR primers and amplicon sizes are given in Table 1. PCR products were verified by sequence analysis.

Total RNA was isolated from the tissues, treated with DNaseI to remove contaminating cellular DNA, and re-purified from the DNaseI. To synthesize cDNA, the RNA was incubated at 45° C with Moloney murine leukemia virus reverse transcriptase as well as poly(dT)15 and random hexamers as primers. To control for variability in mRNA integrity and reverse transcriptase efficiency between tissue samples, the amount of cDNA synthesized for each sample was quantified by trace radiolabeling of a parallel cDNA synthesis reaction carried out in the presence of [α-^{32}P]dCTP. Incorporated and total radiolabel amounts were measured in triplicate by trichloroacetic acid (TCA)-precipitation and scintillation counting. Calculation of the total mass of cDNA synthesized was based on the molar amount of nucleotides present in the reaction converted to mass and multiplied by the ratio of incorporated-to-total radiolabel. Each Q-PCR assay employed 1 ng cDNA as template. Hence, the quantitation of cDNA mass synthesized for each tissue sample served as the normalization control across the tissue board.

Construction of serial dilution standard curves of each specific PCR product were included in every experiment and allowed calculation of transcript copy numbers in the unknown samples by regression analysis. The amount of each template required for the standard curves was determined in a similar manner as described above by trace radiolabeling with [α-^{32}P]dCTP incorporation during the PCR amplication process. The mass of PCR product synthesized was converted to copy number according to the molecular weight of the specific amplicon's size in base pairs. The standard curves covered 8 orders-of-magnitude. All standards and unknown samples were assayed in triplicate.

Analyses of gene expression in breast tissues and correlations with clinicopathological factors.

Using the real-time Q-PCR assays, we determined mRNA levels of ER, ERR, and ErbB family members in a panel of 38 clinical random primary breast carcinomas and normal MECs derived from 9 separate mammoplastic reductions. Each gene was evaluated for alterations in mRNA expression between tissue groups by the non-parametric Kruskal-Wallis ANOVA (KW statistic). Comparisons between tissue groups included normal MECs versus all tumors, ER-LB positive versus negative tumors, and PgR-LB positive versus negative tumors. ER, ErbB, and

ERR family member mRNA levels in each tissue group are depicted as copy number per ng cDNA in Figure 1, Figure 2, and Figure 3, respectively. Analysis of whether correlations existed among pairwise combinations of gene expression levels and clinicopathological factors were performed by the non-parametric Spearman's Rank Correlation Coefficient, designated ρ_s (Spearman's rho). Absolute values of ρ_s near 1 or 0 indicate a strong or weak correlation, respectively, while the sign indicates a positive or negative relationship. Because the DNA ploidy data were in the form of dichotomous observations, tumors containing aneuploid or diploid nuclei were assigned ranks of 1 and 2, respectively. Raw data were used for ranking all other parameters and mRNA levels. Spearman's Correlation Coefficients for all comparisons are shown in Table 2.

The median ERα mRNA levels were approximately 14-fold higher in all breast carcinomas as a group compared to normal MECs (KW = 9.33, $p = 0.002$; Figure 1A). Thus, the Q-PCR assays showed that ERα levels are usually low in normal mammary epithelium and high in breast tumors. This alteration in ERα expression is likely an important etiologic event in breast carcinogenesis (9-14) [reviewed in (15)]. Moreover, the fold-difference of the median ERα mRNA expression was larger when comparing the tumors subgrouped by ER-LB and PgR-LB status. ERα mRNA abundance was 34-fold greater in ER-LB positive versus negative tumors (KW = 16.09, $p < 0.0001$; Figure 1A). Since PgR is a target gene of ERα, PgR-LB-positive status indicates the presence of functional ERα. Hence, ERα mRNA expression was approximately 31-fold greater in PgR-LB-positive tumors compared to negative tumors (KW = 14.90, $p = 0.0001$; Figure 1A). ERα mRNA levels showed a strong and significant correlation with ER protein levels ($\rho_s = 0.859$, $p < 0.0001$; Table 2) and PgR protein levels ($\rho_s = 0.684$, $p < 0.0001$; Table 2) as determined by ligand binding assays. This strong correlation between ERα mRNA levels and ER-LB- as well as PgR-LB-status provides validation of the real-time Q-PCR assays.

ERβ mRNA levels were not significantly increased in the tumor group compared to the normal MEC group. However, 4 of 38 tumors exhibited ERβ expression at levels greater than 3 interquartile regions (IQRs) above median levels. Thus, a subset of breast cancers overexpressed ERβ. The median level of ERβ mRNA expression was approximately 3.2-fold higher in PgR-LB-negative tumors compared to positive tumors (KW = 4.21, $p = 0.040$; Figure 1B). Similar to our results, Dotzlaw *et al.* also found that ERβ mRNA levels were significantly higher in PgR-LB-negative versus positive tumors (16). Hence, ERβ expression was elevated in breast tumors that lacked functional ERα, though ERβ was still expressed at low-to-moderate levels in ERα-LB-positive tumors. Employing immunhistochemistry (IHC), others have reported that ERα-positive tumors are frequently also ERβ positive, (17-19). Several reports indicate that ERβ exhibits prognostic significance in breast cancer, though its role is not clearly defined. Jarvinen *et al.* found that ERβ-positive status by IHC was associated with negative axillary node status, low tumor grade, and low S-phase fraction (18). On the other hand, Jensen *et al.* found by IHC that tumors expressing ERβ in the absence of ERα contained significantly higher levels of proliferation markers than tumors expressing both ERβ and ERα, while ERβ-negative tumors, regardless of ERα status, contained the lowest amounts of proliferation markers (19). Thus, Jensen *et al.* suggested that ERβ-positive status correlated with proliferation in primary breast cancer. These seemingly differing conclusions may be due to Jarvinen *et al.* comparing all ERβ-positive versus all ERβ-negative tumors while Jensen *et al.* further subdivided the ERβ groups as ERα positive or negative. Both studies showed most of the ERβ-positive tumors are also ERα positive. Thus, in the Jarvinen *et al.* study, the correlations between ERβ-positive status and indicators of low tumor aggressiveness were driven by the group being composed of a larger number of ERβ/ERα double-positive tumors. Considering ERα and ERβ are capable of forming functional heterodimers, ERα-ERβ heterodimers may exist in ERα/ERβ double-positive tumors and exhibit differing functional activites than ERβ homodimers in tumors that lack ERα. Both estrogen-bound ERα and ERβ stimulate transcription via an AP-1 site. However, while ERα bound with the antiestrogen tamoxifen inhibits transcription through AP-1 elements, tamoxifen-bound ERβ stimulates transcription via AP-1 sites. Along these lines, Speirs et al. compared tamoxifen-sensitive human breast tumors to tamoxifen-resistant tumors and found ERβ mRNA levels were significantly higher in the resistant group (20). Taken together, it is possible that

ERα-ERβ heterodimers may be associated with a favurable clinical outcome while expression of ERβ homodimers may be associated with poor clinical outcome.

The median EGFR mRNA abundance was approximately 25-fold lower in breast tumors relative to normal MECs (KW = 20.63, $p < 0.0001$; Figure 2A). Within the tumor subgroups, EGFR mRNA median levels were approximately 7.4-fold higher in ER-LB-negative (KW = 13.54, $p = 0.0002$; Figure 2A) and 6.8-fold higher in PgR-LB-negative tumors (KW = 12.40, $p = 0.0004$; Figure 2A) relative to respective positive tumors. EGFR expression levels negatively correlated with ER ($\rho_s = -0.762$, $p < 0.0001$; Table 2) and PgR protein amounts ($\rho_s = -0.633$, $p < 0.0001$; Table 2) as determined by ligand-binding assays. This inverse relationship between EGFR and ERα expression in breast tumors has been previously reported (21-23). In cell culture models, stimulation of ERα transcriptional activity leads to down-regulation of EGFR expression (24), and, conversely, forced overexpression of EGFR leads to estrogen-independent cellular proliferation (25). Moreover, EGFR-positive status has been linked to hormonal therapy resistance (22, 26). Thus, a decrease in EGFR expression concomitant with an increase in ERα expression may be involved in breast tumorigenesis. Alternatively, EGFR overexpression in the absence of ERα expression may also be involved in the etiology of breast cancer.

ErbB2 tended to show an increase in expression, though not statistically significant (KW = 2.90, $p = 0.089$; Figure 2B), when comparing all the breast tumors as a group to the normal MECs. However, approximately 10% of the tumors (4 of 38) expressed ErbB2 mRNA at levels greater than 3 IQRs over the median tumor ErbB2 levels. Moreover, the maximum level of ErbB2 mRNA was approximately 18-fold higher in the tumor group compared to the maximum level in the normal MEC group. Overexpression of ErbB2 in 3 of 4 tumors was associated with ER-LB negative status (Figure 2B). Thus, ErbB2 is overexpressed in a subset of tumors that have a tendency to also lack ERα. Others have reported that ErbB2 is overexpressed, often due to gene amplification, in approximately 10–30% of breast tumors and is a predictor of poor disease outcome (27-29). ErbB2 overexpression is frequently associated with ER-negative status (30). Moreover, ErbB2 overexpression has been linked to tamoxifen resistance (22, 31).

ErbB3 mRNA levels correlated with S-phase fraction ($\rho_s = 0.349$, $p = 0.034$; Table 2), suggesting ErbB3 promotes cellular proliferation. ErbB3 expression also correlated with ERα mRNA levels ($\rho_s = 0.417$, $p = 0.009$; Table 2) and with ErbB2 mRNA levels ($\rho_s = 0.543$, $p = 0.0004$; Table 2). ErbB members form heterodimers as well as homodimers to recruit distinct effector proteins involved in the mitogen-activated protein kinase (MAPK) signaling pathway; ErbB2 is capable of forming heterodimers with EGFR, ErbB3 or ErbB4, and EGFR can heterodimerize with ErbB3 (32-34). The correlation between ErbB2 and ErbB3 expression suggests these members may heterodimerize in breast tumors. In fact, ErbB3 requires a heterodimerization partner to activate signaling (34). Others have also shown a correlation between ErbB3 and ErbB2 expression in breast cancer (35). Additionally, increased expression of ErbB3 has been linked to lymph node metastases (36).

In a similar manner as ErbB2, ErbB4 mRNA abundance showed an inclination to be higher, though not significantly, in all breast tumors compared to normal MECs (KW = 2.99, $p = 0.084$; Figure 2D). However, there was a subset of breast tumors that exhibited ErbB4 overexpression. Approximately 5% (2 of 38) of the tumors exhibited mRNA at levels greater than 1.5 IQRs above the median tumor level, and an additional 5% (2 of 38) at levels greater than 3 IQRs above the median tumor level (Figure 2D). ErbB4 mRNA levels were elevated in ER-LB-positive (KW = 10.55, $p = 0.001$; Figure 2D) and PgR-LB-positive tumors (KW = 13.48, $p = 0.0002$; Figure 2D) relative to the appropriate negative tumors. Further, ErbB4 mRNA levels correlated with ER-LB ($\rho_s = 0.530$, $p = 0.001$; Table 2) and PgR-LB protein levels ($\rho_s = 0.440$, $p = 0.006$; Table 2) as well as with ERα mRNA levels ($\rho_s = 0.739$, $p < 0.0001$; Table 2). Therefore, ErbB4-positive status was associated with the presence of functional ERα. Also, ErbB4 mRNA amounts correlated with ErbB3 mRNA amounts ($\rho_s = 0.416$, $p = 0.009$; Table 2). As with ErbB3, the observed correlations indicate that ErbB4 may activate ERα via MAPK-mediated phosphorylation events. However, ErbB3 and ErbB4 do not heterodimerize (32, 37). Hence the correlation between ErbB3 and ErbB4 may be due to both of these factors independently signaling ERα. As a prognosticator, ErbB4 has been reported to be associated

with more differentiated breast tumor histotypes than the other ErbB members (38) and may serve as favourable biomarker in breast cancer (39, 40).

ERRα showed a tendency to be expressed at approximately 2.3-fold lower levels in the breast tumors compared to the normal MECs, but this difference in expression was not significant (KW = 2.63, $p = 0.10$, Figure 3). However, 4 of 38 tumors overexpressed ERRα; 2 tumors exhibited ERRα mRNA at levels greater than 1.5 IQRs over the median level and an additional 2 tumors showed ERRα levels at greater than 3 IQRs over the median. ERRα mRNA was 2.5-fold higher in the PgR-LB-neg tumor subgroup than in the PgR-LB-pos tumor subgroup (KW = 5.36, $p = 0.021$, Figure 3). This significant increase in ERRα expression suggests an alteration in the role of ERRα in a tumor subgroup that lacks functional ERα. To determine whether ERα or ERRα was the most abundant nuclear receptor of those tested in a particular tissue subgroup, the average mRNA expression levels of the ER and ERR family members were ranked from the most to least abundant and presented in Figure 4. The raw data were log transformed to normally distribute the values with similar variances. Statistical significance between adjacently ranked genes was assessed by 1-way ANOVA with repeated measures on the log-transformed data. As the ANOVA was performed with repeated measures, the expression levels of each gene were paired in each tissue sample by definition. The rank ordering procedure revealed that ERα and ERRα were significantly more abundant than ERβ, ERRβ, and ERRγ in every tissue subgroup. As expected, ERα was the dominant receptor in the complete tumor group as well as in the ER-LB-pos and PgR-LB-pos subgroups. However, in normal MECs and in ER-LB-neg tumors, ERα and ERRα were expressed at equivalent levels. Additionally, ERRα mRNA levels were found to be significantly higher than ERα levels in the PgR-LB-neg tumors ($p = 0.030$). Furthermore, comparisons between ERα and ERRα raw copy numbers by the paired *t* test showed that in the PgR-LB-neg subgroup, ERRα was expressed at higher levels than ERα ($p = 0.050$; Figure5), again suggesting ERRα plays a prominent role in tumors lacking functional ERα.

By Spearman coefficient analysis, ERRα expression levels significantly correlated with ERβ ($\rho_s = 0.349$, $p = 0.032$; Table 2), ErbB2 ($\rho_s = 0.449$, $p = 0.005$; Table 2), and ErbB3 levels ($\rho_s = 0.325$, $p = 0.047$; Table 2). The strong correlation between ErbB2 and ERRα lends evidence toward a functional relationship between these genes. As noted above, ErbB2 overexpression tended to occur in ER-LB-neg tumors, as did ERRα overexpression. Also as noted above, stimulation of MAPK signalling can result in ERα phosphorylation, allowing the unliganded form of ERα to activate transcription. The ErbB2-ERRα correlation may indicate that ERRα is a functional target of ErbB2-mediated stimulation of MAPK signalling. Moreover, the correlations between Erb3 and ERRα and between ErbB3 and ErbB2 indicate that ErbB3 may also potentiate MAPK signalling to activate ERRα.

ERRα-dependent transcriptional activity has been shown to be cell line-specific. Our lab has recently shown that ERRα antagonizes ERα-mediated transcription via an ERRα transcriptional-repression domain specifically in MCF-7 mammary cells (Kraus *et al.*, publication in preparation). On the other hand, Yang and colleagues have shown that ERRα stimulates ERE-dependent transcription in the ERα-neg SK-BR-3 mammary cell line (41, 42). Interestingly, MCF-7 cells contain very low levels of ErbB2 while SK-BR-3 cells contain very high levels of ErB2 (43, 44). Taken together, these observations suggest the following hypothesis: in cells containing low ErbB2 levels, ERRα represses transcription, while in presence of high ErbB2 levels, ERRα activates transcription. This hypothesis predicts that tumors containing high levels of ErbB2 and ERRα would not respond to antiestrogen therapy. Indeed, this hypothesis provides a possible mechanism to explain why ErbB2 has been shown to be an independent marker of tamoxifen resistance (31, 45) and why MCF-7 cells transfected with ErbB2 develop tamoxifen resistance (46). Further, ERRα's phosphorylation state may predict the effectiveness of the therapeutic agent Herceptin, a humanized monoclonal antibody directed against ErbB2; hyperphosphorylated and hypophosphorylated ERRα being indicative of Herceptin-sensitivity and Herceptin-resistance, respectively.

ERRβ mRNA abundance was not significantly different between the tumors and the normal MECs, though 1 of 38 tumors overexpressed ERRβ at levels greater than 3 IQRs above

the median level (Figure 3A). ERRβ showed no significant differences in its median levels among the ER-LB and PgR-LB subgroups. Interestingly, ERRβ expression levels inversely correlated with tumor cell S-phase fraction ($\rho_s = -0.366$, $p = 0.02$; Table 2). The reason for this correlation between ERRβ and S-phase fraction is unknown, but may be suggestive of a role for ERRβ in blocking cellular proliferation or, perhaps, in promoting cellular differentiation. The importance for ERRβ in cellular proliferation and differentiation has been demonstrated by genetic ablation of this locus in the mouse producing a severe defect in placental development leading to lethality at embryonic day 10.5 (47). These ERRβ knockout mice exhibited severely abnormal chorion tissue characterized by an overabundance of trophoblast giant cells and an extreme lack of diploid cells. In our studies, ERRβ mRNA abundance correlated with ERβ mRNA abundance in the breast tumors ($\rho_s = 0.575$, $p = 0.0002$; Table 2), suggesting a possible functional relationship between these genes. It should be noted that ERRβ mRNA levels were quite low but detectable in breast tissues (Figure 3A). Likewise, ERβ mRNA levels were also quite low compared to ERα (Figure 1B and 1A, respectively). Evidently, low ERβ mRNA amounts are sufficient to result in biologically significant protein levels as illustrated by other researcher's IHC-based studies (17-19). As discussed above, ERβ expression has been linked to multiple clinicopathological factors: coexpression of both ERβ and ERα may indicate low tumor aggressiveness (18) whereas expression of ERβ in the absence of ERα may indicate high tumor aggressiveness (19). Furthermore, ERβ mRNA overexpression has been associated with tamoxifen resistance (20). In light of the inverse relationship between ERRβ and S-phase fraction, ERRβ may serve as a marker of low tumor aggressiveness. Conversely, the correlation between ERRβ and ERβ suggests that coexpression of both receptors may serve as a marker of low or high tumor aggressiveness, depending, respectively, upon whether ERα is also coexpressed. Future studies are needed to explore this potential relationship between ERRβ and ERβ and whether ERRβ status would serve as a favorable or unfavorable breast cancer biomarker.

The median mRNA level of ERRγ was elevated approximately 3.1-fold in the breast tumors compared to normal MECs (KW = 9.72, $p = 0.002$; Figure 3B). Thus, overexpression of ERRγ may be important in the development of breast cancer. ERRγ mRNA levels were not significantly elevated in ER-LB-positive tumors versus negative tumors. However, 5 of 6 tumors that overexpressed ERRγ were also ER-LB positive (Figure 3B). Thus ERRγ overexpression or positive status shares a relationship with ERα functionality. Lending indirect support to this supposition, ERRγ expression correlated with ErB4 expression ($\rho_s = 0.325$, $p = 0.049$; Table 2) and, as discussed above, ErbB4 and ERα expression correlated. The median level of ERRγ expression was approximately 2.2-fold higher in tumors typed as diploid (KW =, $p = 0.035$; data not shown). Further, ERRγ mRNA levels positively correlated with diploid status and negatively correlated with aneuploid status ($\rho_s = -0.350$, $p = 0.033$; Table 2). As with ERRβ, the reason for the relationship between ERRγ and DNA ploidy is unknown. As noted above, ErbB4 overexpression has been associated with more histologically differentiated tumors (38) and favorable biomarkers (39, 40). Our findings of an inverse relationship between ERRγ and DNA ploidy along with the positive relationship between ERRγ and ErbB4 suggest that ERRγ-positive status may indicate a less aggressive tumor phenotype.

Cluster analysis facilitated the ordering of similar gene expression patterns in the normal MECs and breast tumors (Figures 6A and 6B, respectively). Tree diagrams similar to those in sequence and phylogenetic analyses were computed based on pairwise correlations in gene expression. Nodes connect the most similar pairs of genes and the length of tree branches represents varying similarities in adjacently listed genes. The ordering of gene expression profiles was performed using Cluster and TreeView software written by Michael Eisen (48). To perform the cluster analysis, the raw data were $\log_2$ transformed and centered on each gene's median expression level. The ordered data table is graphically depicted by colored cells representing gene expression levels; genes that remained unchanged are colored black (log ratios of 0), genes that increased in expression (increasingly positive log ratios) are colored in increasing intensities of red, and genes that decreased in expression (increasingly negative log ratios) are colored in increasing intensities of green. In the normal MECs, cluster analysis

indicated a relationship among ErbB2, ERRα, EGFR and ERα and a relationship among ErbB4, ErbB3 and ERRγ (Figure 6A). In the breast tumors, cluster analysis indicated a relationship among ErbB3, ErbB2, ERRα, and EGFR; a second relationship among ErbB4, ERα, and ERRγ; and a third relationship between ERRβ and ERβ (Figure 6B). Interestingly, these results indicate that ErbB2 and EGFR may signal to ERα in normal MECS, whereas in the tumors, ErbB4 may play a more important role in signaling to ERα. In both tissue groups, ERRα clustered with ErbB2, again indicating a common functionality between these genes. These results also suggest that overexpression of ERRα may be a negative prognostic indicator.

Task 1C. Examine wild-type ERα/tamoxifen-resistant breast tumors (months 25-36).

Examination of tamoxifen-resistant breast tumors had been scheduled to be accomplished following the completion of the analysis of random primary breast tumors as described in Task 1B. I had originally arranged with Dr. Gary Clark to obtain these tamoxifen-resistant tumors from the SPORE at Baylor. Unfortunately, as noted above, the tissue repository was unsalvageable following severe flood damage. Hence, it is not possible to accomplish Task 1C unless another source of these samples is secured. Since it is unlikely to obtain these extremely rare and valuable tamoxifen-resistant fresh frozen tissue within the time period of this fellowship, I propose that Task 1C be removed from the proposal's statement of work.

Specific Aim II and Specific Aim III

Aims II and III had been revised as discussed in the year 1 interim report. Alterations to the tasks associated with these aims have already been approved in a updated Statement of Work.

Revised Specific Aim II - To test whether c-*erb*B2 induced activation of ERRα-dependent transcription may contribute to the TAM-resistant phenotype by generating and characterizing MCF-7 sublines stably-transfected with plasmids inducibly expressing wild-type or dominant-negative ERRα variants in the presence and absence of activated c-*erb*B2.

Revised Task II. Establishment MCF-7 cell sublines that inducibly express ERRα variants in the presence and absence of activated c-*erb*B2, thereby facilitating examination of ERRα's potential role in a defined TAM-resistant cell line model system (months 15-36).

Revised Task 2A. Establishment of MCF-7 cell sublines inducibly expressing ERRα variants in the presence and absence of activated c-erbB2 (months 15-24).

Establishment of cell lines that inducibly express ERRα variants is currently underway but not yet ready to be reported.

Revised Task 2B. Examination of ERRα's potential role in c-*erb*B2-mediated TAM-resistant MCF-7 sublines (months 25-36).

This task has yet to be accomplished.

Revised Task 2C. Characterization of ERRα's phosphorylation status (months 25-36).

This task has yet to be accomplished.

Revised Specific Aim III -To Begin to Elucidate Mechanisms By Which ERRα May Play Roles in Breast Carcinogenesis By Identifying Estrogen-responsive Breast Cancer Prognosticator Genes Which Are Transcriptionally Modulated Through ERRα and Determining the Effects of ERRα on Transcriptional Regulation of These Genes.

Revised Task 3A. Characterization of putative ERRα-binding sites by GMSAs and immunoshift assays (months 25-36).

Identification of multiple genes whose promoters contain ERRα-response elements by GMSAs has been discussed in the year 1 interim report.

Revised Task 3B. Evaluation of ERRα-binding sites using heterologous reporter genes (months 25-36).

This task has yet to be accomplished.

KEY RESEARCH ACCOMPLISHMENTS

- ERRα was overexpressed in approximately 10 % of the breast tumors (Figure 3A).
- ERRα expression was significantly elevated in PgR-LB-neg tumors compared to positive tumors (Figure 3A).
- ERRα mRNA levels were greater than ERα, ERβ, ERRβ, and ERRγ levels in PgR-LB-neg tumors (Figure 4 and Figure 5).
- ERRα expression correlated with ErbB2, ErbB3, and ERβ expression in breast tumors by Spearman coefficient analysis (Table 2).
- ERRα expression patterns ordered with ErbB2, ErbB3 and EGFR expression by cluster analysis (Figure 6).

REPORTABLE OUTCOMES

Publications

Ariazi, E. A., Clark, G.M., and Mertz, J. E. Estrogen-related Receptors: Possible Novel Biomarkers in Breast Cancer (manuscript in preparation).

Patents

Mertz, J. E. and Ariazi, E. A. Estrogen-related Receptors as Biomarkers in Breast Cancer . (U.S. Patent Application in preparation).

Awards

A travel award to the 2001 Gordon Research Conference on Hormone Action was granted.

CONCLUSIONS

Real-time Q-PCR assays have been developed and used to study ERRα mRNA expression in 38 clinical random breast cancers and normal MECs from 9 individuals. ERRα was overexpressed in approximately 10 % of the tumors. ERRα expression was significantly elevated in PgR-LB-neg tumors compared to positive tumors. ERRα mRNA levels were significantly greater than ERα, ERβ, ERRβ and ERRγ levels in PgR-LB-neg tumors. These findings suggest ERRα plays a more prominent role in tumors lacking functional ERα than in other tumors. ERRα expression significantly correlated with ErbB2, ErbB3 and ERβ expression by Spearman coefficient analysis. ERRα expression patterns were ordered with ErbB2, ErbB3 and EGFR expression patterns by cluster analysis. These findings suggest that ERRα may be a target of ErbB signaling. Our lab has recently demonstrated that ERRα represses ERα-mediated transcription via EREs specifically in MCF-7 cells, an ErbB2-negative cell line; while others have shown that ERRα activates transcription independent of ERα in SK-BR-3 cells, an ErbB2-positive cell line. ErbB members signal via the MAPK pathway and ErbB2 has been associated with tamoxifen resistance. Taken together, the following hypothesis was developed: ERRα antagonizes transcription in tumors containing low MAPK activity, and conversely, ERRα potentiates transcription independent of ERα in tumors containing high MAPK activity due to overexpressed ErbB2, ErbB3, and/or EGFR Therefore, high levels of ERRα, or potentially phosphorlyated isoforms of ERRα may indicate resistance to hormonal and/or ErbB2-based therapies.

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APPENDICES

Table 1. Real-time Q-PCR Primer Sets.

ERα	Product = 100 bp
5'ERα	5'-GGA GGG CAG GGG TGA A-3'
3'ERα	5'-GGC CAG GCT GTT CTT CTT AG-3'
ERβ	Product = 137 bp
5'ERβ	5'-TTC CCA GCA ATG TCA CTA ACT T-3'
3'ERβ	5'-TTG AGG TTC CGC ATA CAG A-3'
EGFR	Product = 104 bp
5'EGFR	5'-GTG ACC GTT TGG GAG TTG ATG A-3'
3'EGFR	5'-GGC TGA GGG AGG CGT TCT C-3'
ErbB2	Product = 82 bp
5'ErbB2	5'-GGG AAG AAT GGG GTC GTC AAA-3'
3'ErbB2	5'-CTC CTC CCT GGG GTG TCA AGT-3'
ErbB3	Product = 106 bp
5' ErbB3	5'-GTG GCA CTC AGG GAG CAT TTA-3'
3' ErbB3	5'-TCT GGG ACT GGG GAA AAG G-3'
ErbB4	Product = 105 bp
5' ErbB4	5'-TGC CCT ACA GAG CCC CAA CTA-3'
3' ErbB4	3'-GCT TGC GTA GGG TGC CAT TAC-3'
ERRα	Product = 100 bp
5'ERRα	5'-AAA GTG CTG GCC CAT TTC TAT-3'
3'ERRα	5'-CCT TGC CTC AGT CCA TCA T-3'
ERRβ	Product = 144 bp
5' ERRβ	5'-TGC CCT ACG ACG ACA A-3'
3' ERRβ	5'-ACT CCT CCT TCT CCA CCT T-3
ERRγ	Product = 67 bp
5'ERRγ	5'-GGC CAT CAG AAC GGA CTT G-3'
3'ERRγ	5'-GCC CAC TAC CTC CCA GGA TA-3'

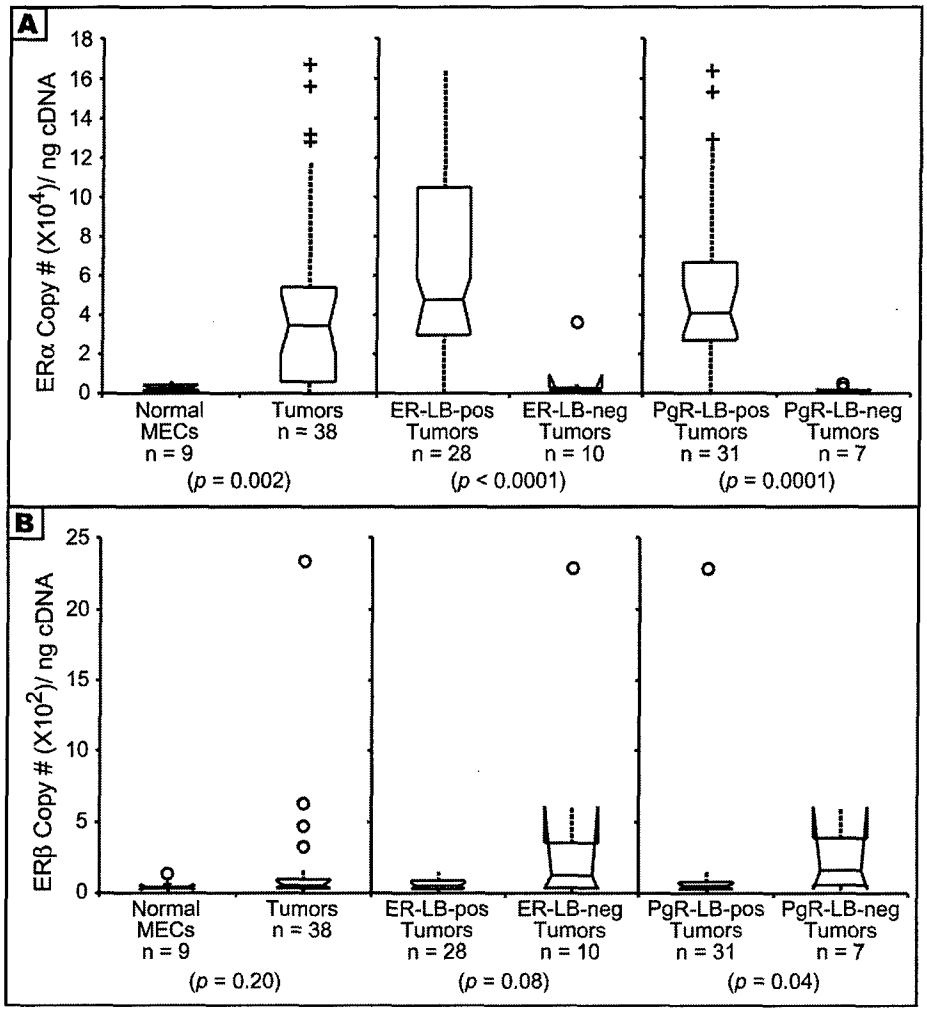

Figure 1. ER family member mRNA levels in breast tissues. (A) ERα levels. (B) ERβ levels. The notched box shows the median, lower and upper quartiles, and confidence interval around the median. The dotted-line connects the nearest observations within 1.5 inter-quartile ranges (IQRs) of the lower and upper quartiles. Far outliers, over 3.0 IQRs away, are denoted with a ○, and near outliers, between 1.5 and 3.0 IQRs away, are denoted with a +. Statistical significance determined by the non-parametric Kruskal-Wallis (KW) ANOVA.

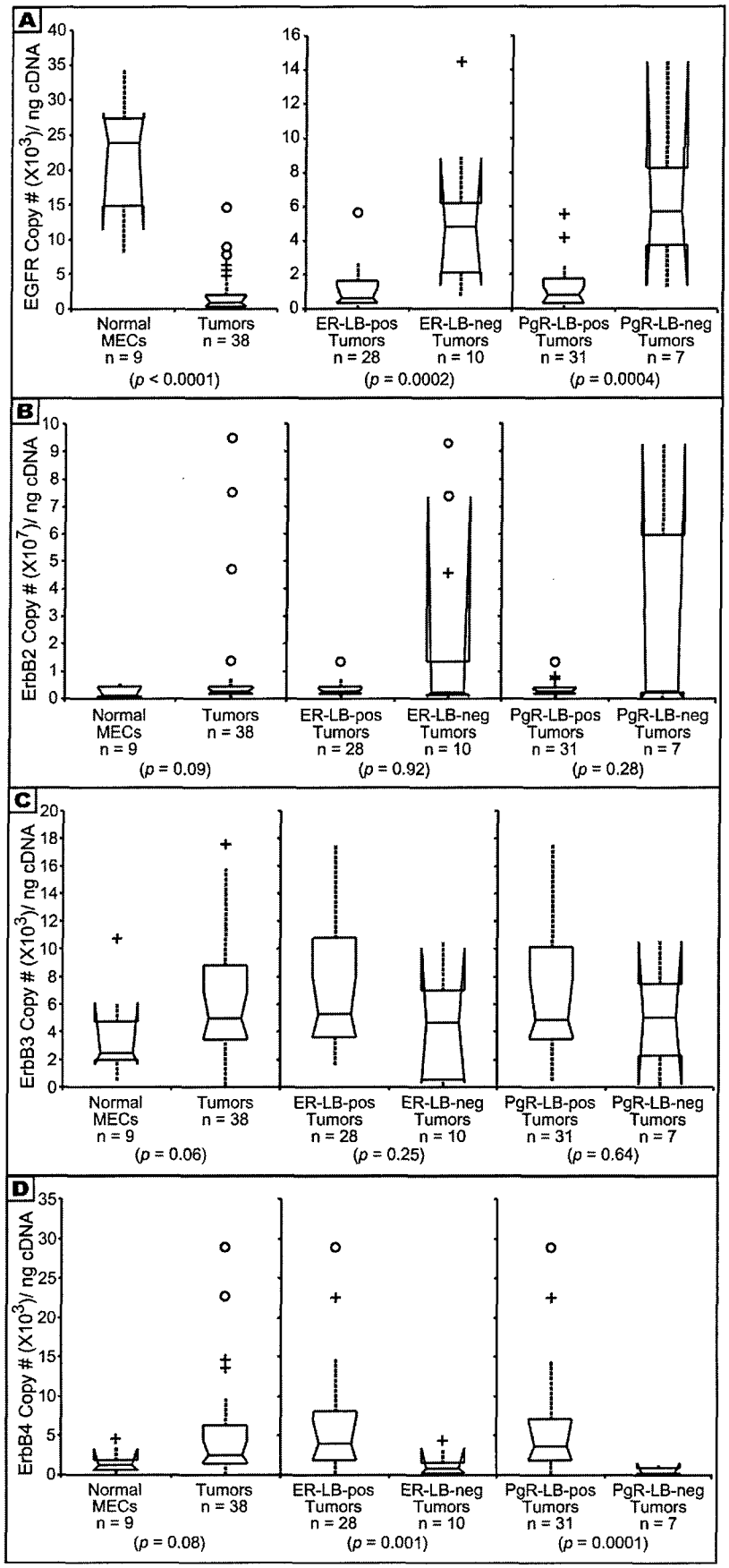

Figure 2. ErbB family member mRNA levels in breast tissues. (A) EGFR levels. (B) ErbB2 levels. (C) ErbB3 levels. (D) ErbB4 levels. The notched box shows the median, lower and upper quartiles, and confidence interval around the median. The dotted-line connects the nearest observations within 1.5 inter-quartile ranges (IQRs) of the lower and upper quartiles. Far outliers, over 3.0 IQRs away, are denoted with a ○, and near outliers, between 1.5 and 3.0 IQRs away, are denoted with a +. Statistical significance determined by the non-parametric Kruskal-Wallis (KW) ANOVA.

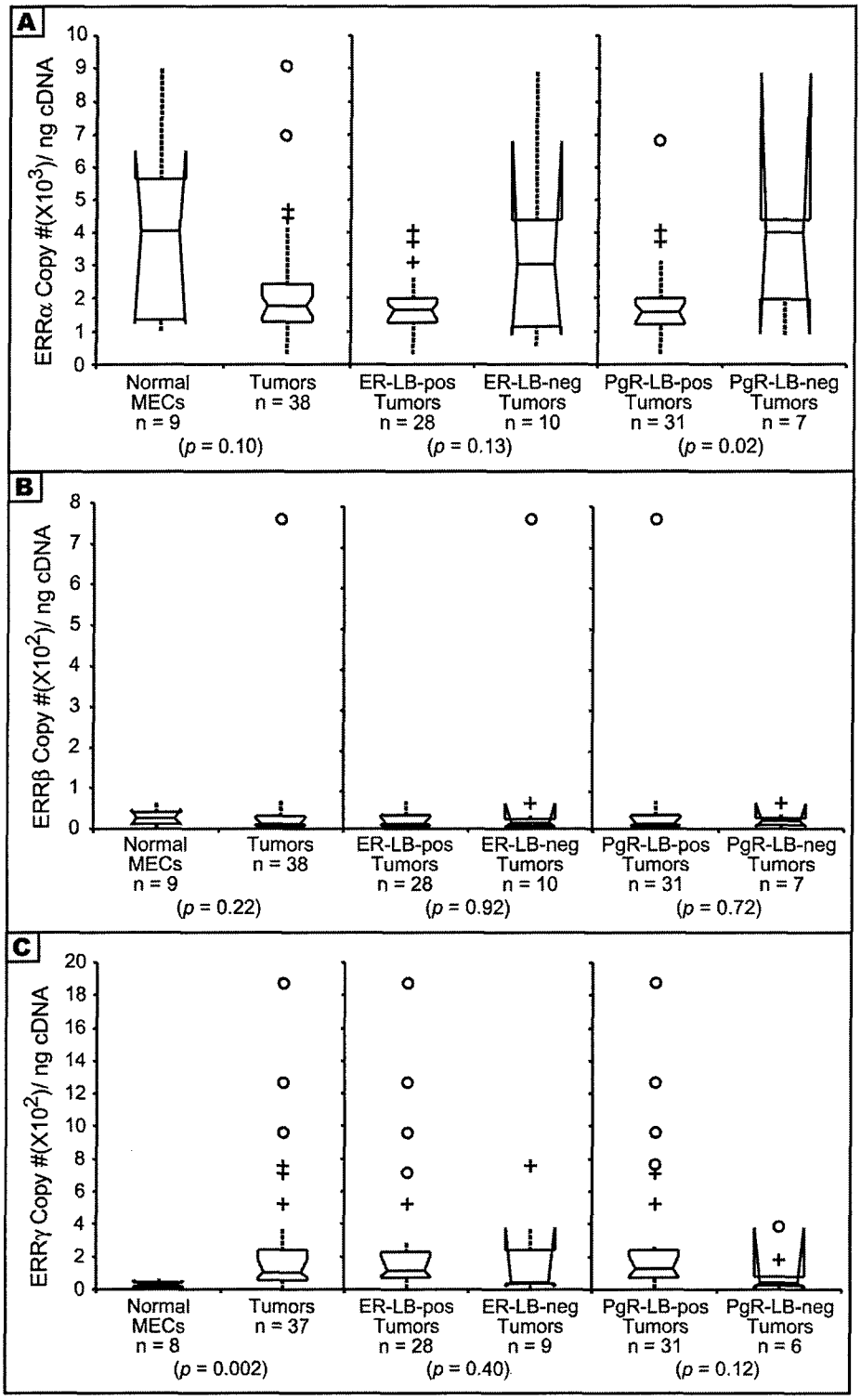

Figure 3. ERR family member mRNA levels in breast tissues. (A) ERRα levels. (B) ERRβ levels. (C) ERRγ levels. The notched box shows the median, lower and upper quartiles, and confidence interval around the median. The dotted-line connects the nearest observations within 1.5 inter-quartile ranges (IQRs) of the lower and upper quartiles. Far outliers, over 3.0 IQRs away, are denoted with a ○, and near outliers, between 1.5 and 3.0 IQRs away, are denoted with a +. Statistical significance determined by the non-parametric Kruskal-Wallis (KW) ANOVA.

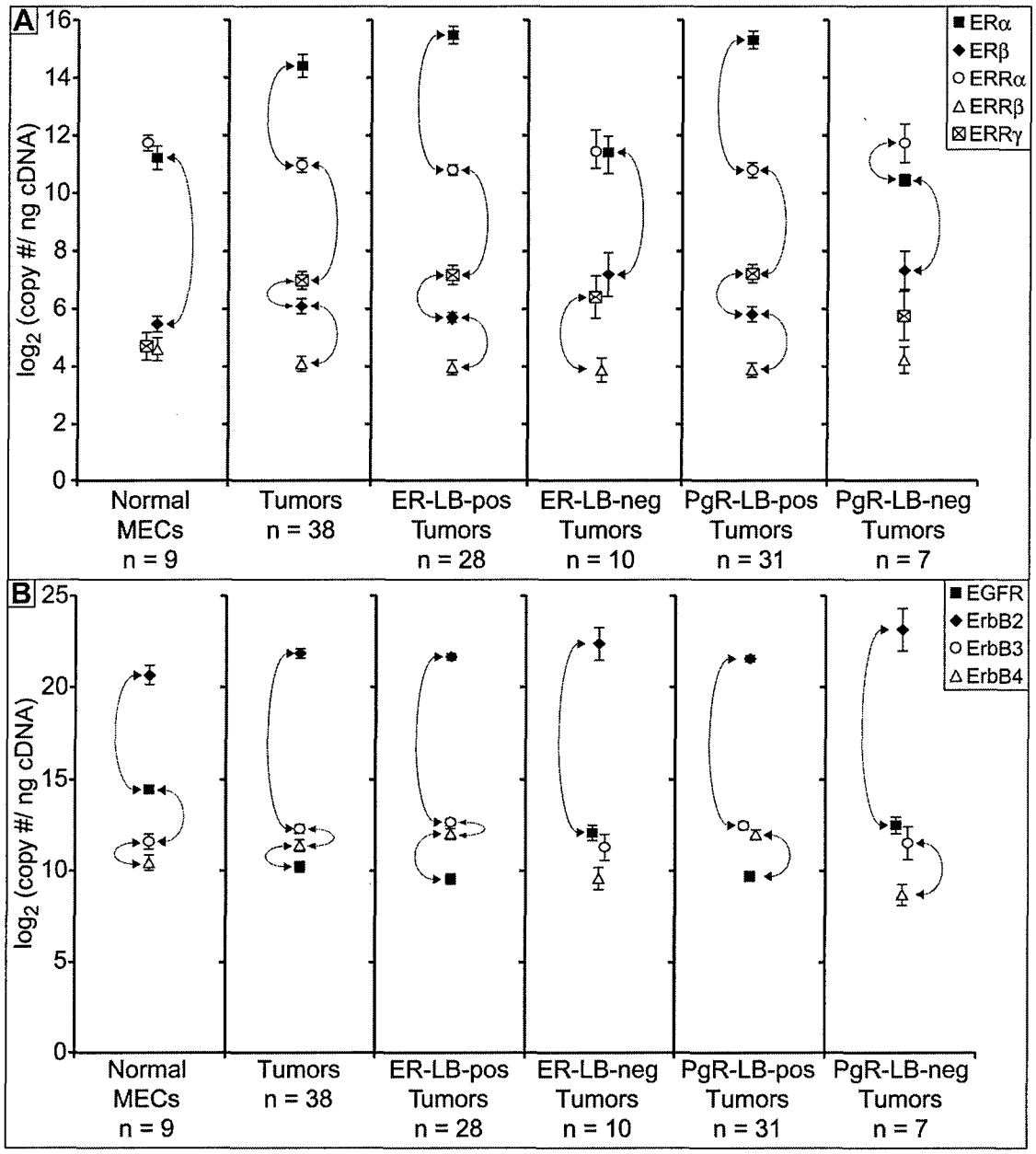

Figure 4. Rank-order of mRNA expression. (A) ER and ERR family members. (B) ErbB family members. Gene expression values were log transformed to normally distribute the data. Genes were ranked in descending order according to their mean expression levels and presented along with their corresponding standard errors. Significance was assessed between pairs of adjacently ranked genes by 1-way ANOVA with repeated measures. Significant differences in gene expression levels at $p < 0.05$ are indicated by arrows.

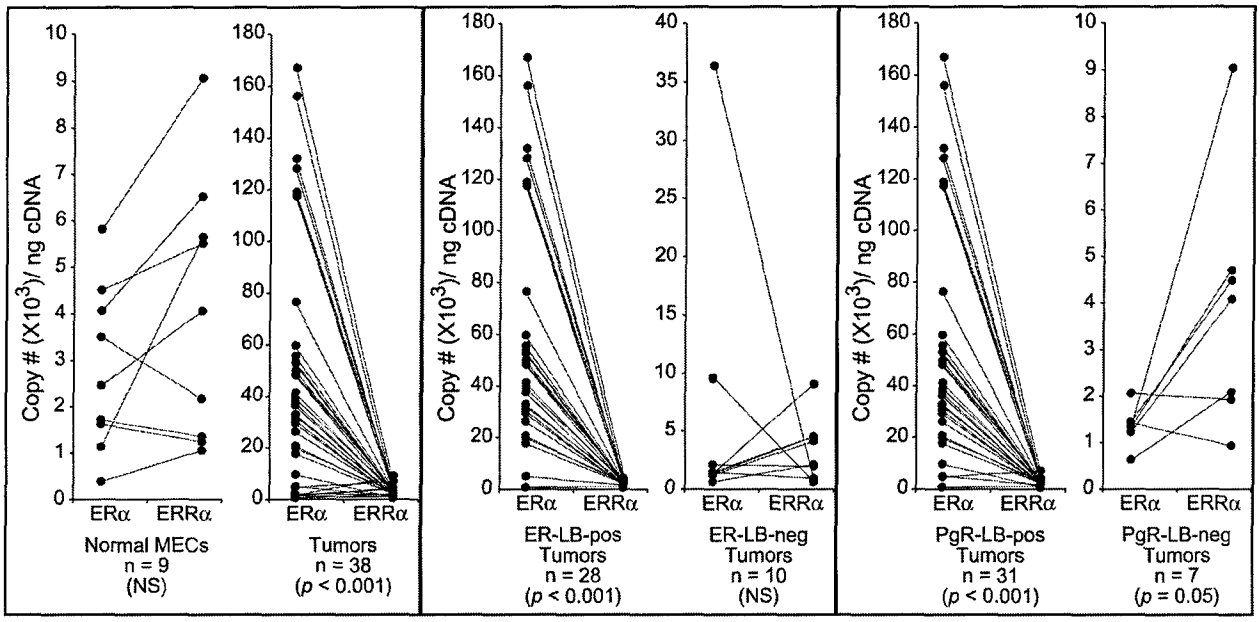

Figure 5. Comparison between ERα and ERRα mRNA levels within the same tissue sample. Significance assessed by the paired *t* test.

Table 2. Spearman's Rank Correlation Coefficients (ρ_s) for breast carcinoma properties. The coefficients, p-value, and number of observations are listed on the top, middle, and bottom lines, respectively. Significant correlation coefficients are bolded and marked with an asterisk.

		PgR-LB	S-phase	DNA Ploidy	ERα	ERβ	EGFR	ErbB2	ErbB3	ErbB4	ERRα	ERRβ	ERRγ
ER-LB		**0.737***	-0.070	0.090	**0.859***	-0.109	**-0.762***	-0.006	0.173	**0.530***	-0.231	0.109	0.149
		< 0.0001	0.68	0.59	**< 0.0001**	0.51	**< 0.0001**	0.97	0.30	**0.001**	0.16	0.51	0.38
		39	38	39	**38**	38	**38**	38	38	**38**	38	38	37
PgR-LB			-0.132	0.069	**0.684***	-0.220	**-0.633***	0.051	0.217	**0.440***	-0.145	0.120	0.045
			0.43	0.68	**< 0.0001**	0.18	**< 0.0001**	0.76	0.19	**0.006**	0.39	0.47	0.79
			38	39	**38**	38	**38**	38	38	**38**	38	38	37
S-phase				**0.750***	-0.134	0.010	-0.088	0.082	**0.349***	-0.177	0.190	**-0.366***	-0.302
				< 0.0001	0.43	0.95	0.60	0.63	**0.034**	0.29	0.26	**0.026**	0.07
				39	37	37	37	37	**37**	37	37	**37**	36
DNA Ploidy					0.062	0.048	-0.236	0.125	0.190	-0.039	0.192	-0.187	**-0.350***
					0.71	0.77	0.15	0.45	0.25	0.82	0.25	0.26	**0.033**
					38	38	38	38	38	38	38	38	**37**
ERa						-0.145	**-0.536***	0.171	**0.417***	**0.739***	-0.128	0.073	0.179
						0.39	**0.001**	0.31	**0.009**	**< 0.0001**	0.44	0.66	0.29
						38	**38**	38	**38**	**38**	38	38	37
ERβ					0.267		0.078	0.243	-0.152	-0.160	**0.349***	**0.575***	-0.131
					0.49		0.64	0.14	0.36	0.34	**0.032**	**0.0002**	0.44
					9	38	38	38	38	38	**38**	**38**	37
EGFR					**0.733***	0.033		0.216	0.094	-0.296	0.192	-0.130	-0.108
					0.025	0.93		0.19	0.57	0.07	0.25	0.44	0.52
					9	9	38	38	38	38	38	38	37
ErbB2					**0.817***	0.250	**0.883***		**0.543***	0.043	**0.449***	-0.103	0.120
					0.007	0.52	**0.002**		**0.0004**	0.80	**0.005**	0.54	0.48
					9	9	9	38	**38**	38	**38**	38	37
ErbB3					0.517	0.150	0.483	**0.700***		**0.416***	**0.325***	-0.280	0.156
					0.15	0.70	0.19	**0.036**		**0.009**	**0.047**	0.09	0.36
					9	9	9	**9**	**38**	**38**	**38**	38	37
ErbB4					0.350	0.267	-0.150	0.200	0.600		-0.152	-0.097	**0.325***
					0.36	0.49	0.70	0.61	0.09		0.36	0.56	**0.049**
					9	9	9	9	9	**38**	38	38	**37**
ERRα					**0.700***	0.333	**0.900***	**0.933***	0.567	0.050		0.296	0.034
					0.036	0.38	**0.0009**	**0.0002**	0.11	0.90		0.07	0.84
					9	9	**9**	**9**	9	9	**38**	38	37
ERRβ					0.233	0.267	0.500	0.583	0.283	0.000	**0.767***		-0.090
					0.55	0.49	0.17	0.10	0.46	1.00	**0.016**		0.60
					9	9	9	9	9	**9**	**38**	37	37
ERRγ					0.476	0.333	0.143	0.643	**0.810***	**0.762***	0.381	0.167	
					0.23	0.42	0.74	0.09	**0.015**	**0.028**	0.35	0.69	
					8	8	8	8	**8**	**8**	8	8	

Breast Tumors

Normal Mammary Epithelial Cells

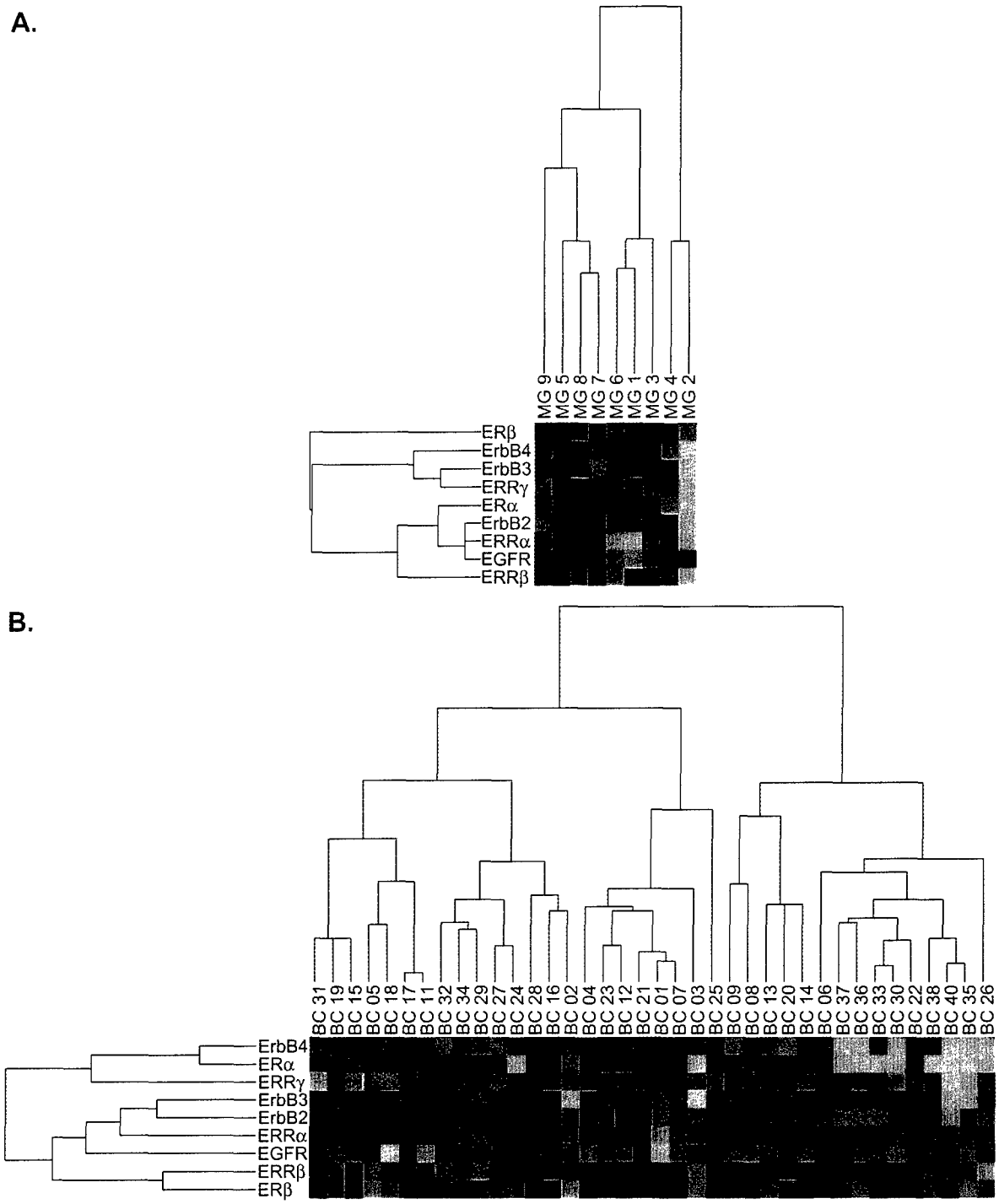

Figure 6. Cluster analysis. (A) Normal MECs. (B) Random Primary Breast Tumors. Tree diagrams represent similarities in gene expression patterns. Gene expression levels unchanged from the median level are displayed as black, elevated expression levels are displayed in increasing intensities of red, and reduced expression levels in increasing intensities of green.

DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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